

A rapid, sensitive enrichment PCR to detect *Salmonella* and ETEC infections in pigs

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Abstract

Salmonella and *Escherichia coli* infections, particularly enterotoxigenic *E. coli* (ETEC), are a problem in piglet production. In addition, *Salmonella* is a major concern in the pork industry as a carrier state can be induced post infection allowing the pathogen to spread across the pig herd, onto many carcasses at slaughter and into the human food chain. Detection methods for these pathogens are currently highly laborious, with *Salmonella* detection taking over 5 days to give a verified positive result. Identification of ETEC isolates involves detecting the presence of toxin or fimbriae (or their genes by PCR), particularly F4, the most common fimbriae in piglet ETEC infection. Alternative methods such as real time PCR are unable to detect low levels of infection directly from the sample. Enrichment PCR assays have been successfully implemented for the detection of pathogens in foodstuffs, however, faecal material often is inhibitory to PCR based methods.

The enrichment PCR assay presented here overcomes these problems and allows the rapid detection of *Salmonella* and ETEC from piglet faeces. Faecal samples are pre-enriched in buffered peptone water and DNA is extracted, followed by a multiplex PCR. This shortens the detection time for the presence of these organisms to 30 h. The multiplex PCR uses primers for detection of both the invasion gene (*invA*) in *Salmonella* required for enteric infection, and the F4 fimbriae of ETEC. Spiked faecal samples have shown a detection limit of <10 cells of either pathogen per 1g faeces. Moreover, pathogens at low levels have been detected on farm. This assay will facilitate early detection of enteric infections, and subsequently enable monitoring of the pathogen status of the pigs until all low level carriage has been eliminated. This has strong potential to control pathogen spread within pig herds, and thus the human food chain.

Introduction

Piglets undergo a growth check at weaning as a result of diet change, social stresses and increased pathogen susceptibility : as a consequence substantial losses occur during this time (Lalles *et al.*, 2007). The foremost bacterial pathogens at weaning are *Escherichia coli* and *Salmonella*, both of which cause serious enteric infections that result in dehydration, profuse diarrhoea and sometimes death (Lalles *et al.*, 2007). Often pathogens can exist as co-infections, resulting in increased infection severity (Melin *et al.*, 2004). During infection *Salmonella* penetrates the intestinal barrier, causes necrotising enterocolitis and is spread system wide (Letellier *et al.*, 2000). This results in a carrier state being induced where *Salmonella* can be carried, and therefore excreted, by the animal for up to 28 weeks (Wood and Rose, 1992). In addition, enterotoxigenic *E.coli* (ETEC) causes significant financial losses in the pork industry due to infections in young pigs. ETEC infection is induced by a heat labile or heat stable toxin switching on electrolyte secretion into the gut lumen (Lalles *et al.*, 2007). Fimbriae are required for adherence of ETEC to the gut epithelium and for toxin delivery (Lalles *et al.*, 2007). The toxin and fimbriae types present in infection vary, however, research suggests the heat labile toxin and F4 are the most predominant factors in post-weaning piglets (Osek, 1999). As these pathogens can be transferred to the carcass at slaughter, there is a large amount of emphasis placed on the “farm to fork” strategy to control zoonotic transmissions into meat.

Conventional methods for identification of these pathogens include isolation of a strain with subsequent biochemical, serological or gene detection tests. *E.coli* isolates are selected from diagnostic media whereas *Salmonella* requires a three stage enrichment process with confirmatory tests, taking over 5 days.

Both of these “gold standard” methods are laborious and time consuming, and involve screening many negative results and for ETEC, exhibit limited sensitivity.

More recently the rapid method of polymerase chain reaction (PCR) has been successfully employed to identify pathogenicity genes of these organisms without requiring isolation of the organism itself. Although successful in food (Ferretti *et al.*, 2001), faecal matter has been more problematic due to the presence of PCR inhibitors and a high background flora (Widjoatmodjo *et al.*, 1992). PCR detection directly from faeces can detect as low as 10^5 c.f.u. g^{-1} of the organism of interest (Kongmuang *et al.*, 1994). However, PCR enrichments for *Salmonella* in faeces have shown detection limits of 10^2 c.f.u. g^{-1} , proving it is a viable alternative to culture (Chiu and Ou, 1996; Mainar-Jaime *et al.*, 2008). *E.coli* pathogenicity genes are often detected in a multiplex PCR that allows identification of all types of pathogenic *E.coli* but still requires strain isolation first with detection directly from faeces rarely considered.

The method presented here aims to provide a rapid, sensitive detection system for *Salmonella* and F4 positive ETEC to aid surveillance of these pathogens in pig populations. Faeces are enriched for 24 h, DNA extracted and the presence of *Salmonella* and ETEC is detected in a multiplex PCR using previously published primers to produce a 453 bp and a 753 bp product respectively (Arnold *et al.*, 2004; Do *et al.*, 2005).

Material and Methods

Enrichment Multiplex PCR

One g faecal matter was homogenised into 9 mL buffered peptone water. Faecal suspensions were enriched at 37°C, 24 h before being centrifuged at 600 g for 5 min. Then 1 mL supernatant was centrifuged at 13,000 g 5 min. Pellets were washed in 1 mL TE buffer. DNA was extracted from the pellet using the Nucleospin tissue kit (Macherey Nagel, Germany).

PCR reactions used primers F4F and F4R to amplify a 764 bp PCR product of the F4 fimbriae gene of ETEC (Do *et al.*, 2005) and the improved primers, *invAF* and *invAR*, of Arnold *et al.* (2004) to generate a 476 bp PCR product of the plasmid encoded *Salmonella* invasion gene.

PCR products were analysed in 1.5 % agarose gels in 1x TAE buffer (40 mM Tris Acetate pH 8, 1 mM EDTA) run at 70 V 1 h. Bands were visualised by staining gels in ethidium bromide 0.5 $\mu g\ ml^{-1}$, then UV illumination and photographing with Biorad software (Biorad, USA).

Spiking of Enrichment PCR

To determine the sensitivity of the enrichment/multiplex PCR, diluted cultures of *Salmonella* Typhimurium LT2 and ETEC F4 positive isolate Y04124 (VLA, Weymouth) in MRD, were spiked into piglet faeces at concentrations of 1 c.f.u. g^{-1} to 10^4 c.f.u. g^{-1} to represent different levels of infection. One fraction of faeces was always processed unspiked as a negative control.

Conventional *Salmonella* method

Hundred μL from enriched broth (outlined above) was inoculated into 9.9 mL Rappaport Vassiliadis broth and further enriched for 24 h at 42°C. Ten μL was streaked onto XLD (37°C, 24 h). Presumptive positive colonies were re-streaked onto BHI agar and confirmed using an oxidase test and serological testing with Poly O A - S antisera (VLA, Weybridge, UK).

Results

Primers to detect the F4 fimbriae gene of ETEC and the *invA* invasion gene of *Salmonella* were incorporated successfully into the same PCR reaction by modifying the PCR conditions. A representative gel from reference isolates is shown in Figure 1A, Lane b shows the 476 bp product representative of *Salmonella*, lane c the 764 bp product of the F4 gene of ETEC and lane d both ETEC and *Salmonella* as represented by the two bands present.

Salmonella and ETEC cultures were spiked into faeces at different concentrations and the enrichment PCR method used to detect samples after enrichment. When cultures were spiked individually into faeces the method was able to detect the pathogens in all samples, where the lowest level of detection was 2 c.f.u.g⁻¹ for *Salmonella* and 6 c.f.u.g⁻¹ for ETEC (data not shown). Results for the classical enrichments for the spiked *Salmonella* were comparable to the PCR as at all three levels of spiking samples were positive for *Salmonella*. Figure 1B shows detection of both organisms when spiked into faeces in combination at set levels using the enrichment PCR. The PCR was able to detect both organisms at levels of 1 c.f.u. g⁻¹ through to 10⁴ c.f.u. g⁻¹ in both samples (Figure 1B, lanes a-i). The lowest level of detection achieved was 3 c.f.u. g⁻¹ for *Salmonella* and 2 c.f.u.g⁻¹ for ETEC (lane a). However, the enriched PCR was not always successful. The 764 bp band representing the F4 gene of ETEC was often weak, and in one case absent (lane f). However, the enrichment PCR was better than detection by classical methods for the detection of *Salmonella* as in the mixed spiked samples classical methods failed to detect the organism spiked at 1 c.f.u.g⁻¹ in the second faeces sample.

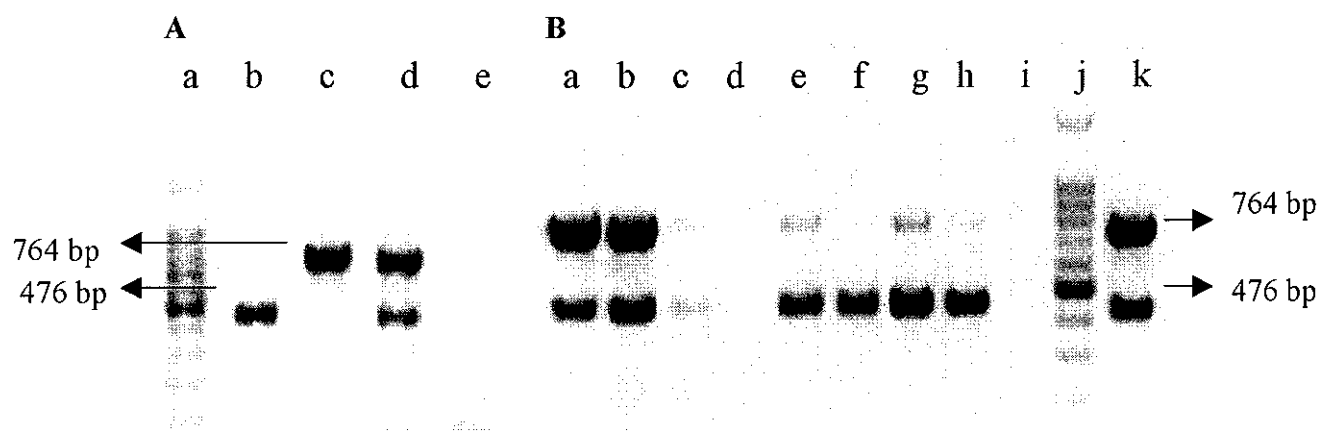


Figure 1.

A Detection of ETEC and *Salmonella* Typhurimum LT2 in multiplex PCR reaction. Lane a) 100 bp ladder, b) *Salmonella* Typhurimum LT2, c) ETEC isolate Y04124, d) *Salmonella* Typhurimum and ETEC isolate Y04124, e) negative control (water).

B Detection of spiked levels of *Salmonella* Typhimurium LT2 and enterotoxigenic *E.coli* isolate Y041424 in replicate samples of piglet faeces. Each sample contains equal quantities of spiked *Salmonella* and ETEC into faeces. Lanes a-d are faeces sample 1, lanes e to i are sample 2. Lane a & e contain 1 c.f.u. g⁻¹ *Salmonella* and ETEC in faeces; lanes b & f 10 c.f.u. g⁻¹ *Salmonella* and ETEC in faeces; lanes c & g 10² c.f.u. g⁻¹ *Salmonella* and ETEC in faeces; lane h 10³ c.f.u. g⁻¹ *Salmonella* and ETEC in faeces; and lanes d & i unspiked faeces. Lane j contained a 100 bp ladder, lane k combined DNA of *Salmonella* Typhurimum LT2 & ETEC wild type isolate Y041424.

Discussion

Our results show that the multiplex PCR described here is able to detect pathogens in faeces, when present together or separately, and present at <10 c.f.u.g⁻¹. This level is lower than previously reported for detection of *Salmonella* by combination enrichment PCR (Chiu and Ou, 1996). The low sensitivity threshold is essential for *Salmonella* detection due to the low level shedding that occurs for several months post-infection (Wood and Rose, 1992). This method sensitivity is similar to typical culture methods but has the advantage taking less than 30 h compared to 5 d for cultured results. We are not able to compare ETEC detection limits with conventional culture methods as identification by PCR or antisera is generally based on a disease isolate, not direct detection from faeces (Osek, 1999). Therefore detection limits are dictated by the dilutions from which the colonies are selected, often exceeding levels of 10⁶ c.f.u. g⁻¹. The

ability to detect low levels of ETEC in faeces is highly advantageous as an indicator of early stages of infection or sub-clinical infections.

The one false negative result for ETEC, (Figure 1B, lane f) may be due to high background levels of *E. coli* not permitting ETEC to grow to detectable levels. False negative results are a problem when detecting pathogens from faeces, although this effect may be minimised with further optimisation (Arnold *et al.*, 2004). However, results for *Salmonella* detection by the classical method indicated the multiplex PCR was more sensitive than existing classical and molecular methods for detection of *Salmonella* from faeces, and also quicker than classical culture. Our results correlate to those previously found where PCR detection of *Salmonella* was more effective by enrichment PCR than by culture (Chiu and Ou, 1996; Mainar-Jaime *et al.*, 2008).

Conclusions

This enrichment multiplex PCR provides a sensitive detection method for *Salmonella* and ETEC, with a detection limit of less than 10 cells g⁻¹ faeces for both organisms. This is a very effective method to study sub-clinical infections and also the low level pathogen shedding associated with *Salmonella* infection. Moreover, detection of *Salmonella* can be completed within 30 h as compared with the five days required for conventional bacterial culture methods, therefore greatly reducing time and labour. This method is currently being applied to large intervention farm trials to aid our surveillance capacity for these pathogens within the pig, and therefore, the pork industry.

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